

07/27/00
JC696 U.S. PTO

07-28-00

A

Certificate of Mailing

Date of Deposit JULY 27, 2000

Label Number: EL509049251US

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to: BOX PATENT APPLICATION, Commissioner for Patents, Washington, D.C. 20231.

Luis Cruz

Printed name of person mailing correspondence

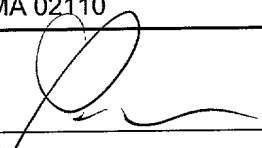
Signature of person mailing correspondence

UTILITY PATENT APPLICATION TRANSMITTAL UNDER 37 CFR §1.53(b)

Attorney Docket Number	04843/080001
Applicant	Ole Isacson et al.
Title	CELL IMPLANTATION THERAPY FOR NEURODEGENERATIVE DISEASES OR DISORDERS
PRIORITY INFORMATION:	
APPLICATION ELEMENTS:	
Cover sheet	1 page
Specification	23 pages
Claims	3 pages
Abstract	1 page
Drawing	4 sheets
Combined Declaration and POA, which is: <input checked="" type="checkbox"/> Unsigned; <input type="checkbox"/> Newly signed for this application; <input type="checkbox"/> A copy from prior application ["**SERIAL NUMBER**"] and the entire disclosure of the prior application is considered as being part of the disclosure of this new application and is hereby incorporated by reference therein.	2 pages
Statement Deleting Inventors	0 pages
Sequence Statement	0 pages
Sequence Listing on Paper	0 pages
Sequence Listing on Diskette	0 disk
Small Entity Statement, which is: <input checked="" type="checkbox"/> Unsigned; <input type="checkbox"/> Newly signed for this application; <input type="checkbox"/> A copy from prior application ["**SERIAL NUMBER**"] and such small entity status is still proper and desired.	1 page
Preliminary Amendment	0 pages

07/27/00
JC696 U.S. PTO

07/27/00

IDS	0 pages
Form PTO 1449	0 pages
Cited References	0 references
Recordation Form Cover Sheet and Assignment	0 pages
Assignee's Statement	0 pages
English Translation	0 pages
Certified Copy of Priority Document	0 pages
Return Receipt Postcard	1
FILING FEES:	
Basic Filing Fee: \$345	\$345.00
Excess Claims Fee: 11 - 20 x \$9	
Excess Independent Claims Fee: 1 - 3 x \$78/\$39	
Multiple Dependent Claims Fee: \$260/\$130	
Total Fees:	\$345.00
<input checked="" type="checkbox"/> Enclosed is a check for \$345.00 to cover the total fees. <input type="checkbox"/> Charge [**AMOUNT**] to Deposit Account No. 03-2095 to cover the total fees. <input type="checkbox"/> The filing fee is not being paid at this time. <input checked="" type="checkbox"/> Please apply any other charges, or any credits, to Deposit Account No. 03-2095.	
CORRESPONDENCE ADDRESS:	
Paul T. Clark Reg. No. 30,162 Clark & Elbing LLP 176 Federal Street Boston, MA 02110	
Telephone: 617-428-0200 Facsimile: 617-428-7045	
Signature 	Date <u>July 27, 2000</u>

Applicant or Patentee : Ole Isacson et al.
 Serial or Patent No. :
 Filed or Issued : July 27, 2000
 Title : CELL IMPLANTATION THERAPY FOR NEURODEGENERATIVE DISEASES OR DISORDERS

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
 (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The McLean Hospital Corporation
Address of Organization: 115 Mill Street, Belmont, MA 02178
Type of Organization:

- ☐ University or Other Institution of Higher Education
☒ Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
☐ Nonprofit Scientific or Educational under Statute of State of the United States of America
 Name of State:
 Citation of Statute:
☐ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) If Located in the United States of America
☐ Would Qualify as Nonprofit Scientific or Educational under Statute of State of the United States of America If Located in the United States of America
 Name of State:
 Citation of Statute:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled CELL IMPLANTATION THERAPY FOR NEURODEGENERATIVE DISEASES OR DISORDERS by inventors Ole Isacson and Kwang Soo Kim described in

- ☒ the specification filed herewith.
☐ application serial no. [**SERIAL NUMBER**], filed [**FILING DATE**].
☐ patent no. [**PATENT NUMBER**], issued [**ISSUE DATE**].

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name:
Address:

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: PETER PASKEVICH, M.D.

Title: Assistant General Director for Research Administration

Address: McLean Hospital, 115 Mill Street, Belmont, MA 02178

Signature: _____ **Date:** _____

Certificate of Mailing

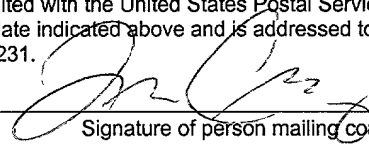
Date of Deposit JULY 27, 2000

Label Number: EL509049251US

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as **"Express Mail Post Office to Addressee"** with sufficient postage on the date indicated above and is addressed to: BOX PATENT APPLICATION, Commissioner for Patents, Washington, D.C. 20231.

Luis Cruz

Printed name of person mailing correspondence



Signature of person mailing correspondence

APPLICATION

FOR

UNITED STATES LETTERS PATENT

APPLICANT : OLE ISACSON AND KWANG SOO KIM

TITLE : CELL IMPLANTATION THERAPY FOR
NEURODEGENERATIVE DISEASES OR DISORDERS

**CELL IMPLANTATION THERAPY FOR NEURODEGENERATIVE
DISEASES OR DISORDERS**

Statement as to Federally Sponsored Research

This invention was sponsored in part by Grant #P50 NS39793-01 from the National Institutes of Health. The Government has certain rights to this invention.

Background of the Invention

The field of the invention is cell implantation therapy for neurodegenerative disorders.

Neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's disease are becoming ever more prominent in our society. A direct approach towards therapeutic treatment of these diseases is through replacement therapy where normal tissue is transplanted back to the nervous system. Recently, significant progress has been achieved with transplants in Parkinson's disease (PD), but the process is heavily dependent on an unstable and problematic source of fetal tissue. Neural stem cells may become the tissue/cell source necessary for developing the therapeutic potential of neural transplantation. Stem cells are self-renewing, multipotent and provide a well-characterized and clean source of transplantable material to replace intrinsic neuronal systems, that do not spontaneously regenerate after injury, such as the dopaminergic (DA) system affected in PD and aging. Current clinical data indicate proof of principle for this cell implantation therapy for PD. Furthermore, the disease process does not appear to negatively affect the transplanted cells, although the patient's endogenous DA

system degeneration continues. The stem cells may themselves be transplanted or, alternatively, they may be induced to produce differentiated cells (e.g., neurons, oligodendrocytes, Schwann cells, or astrocytes) for transplantation.

To date, stem cells have been purified and characterized from several tissues. For example, neural stem cells have been purified from the mammalian forebrain (Reynolds and Weiss, *Science* 255:1707-1710, 1992) and these cells were shown to be capable of differentiating into neurons, astrocytes, and oligodendrocytes. PCT publications WO 93/01275, WO 94/16718, WO 94/10292 and WO 94/09119 describe uses for these cells. Neural stem cells may be used to generate oligodendrocytes and/or astrocytes for use in transplants for the treatment of multiple sclerosis and other myelin-associated diseases (Brustle et al., *Science* 285: 754 (1999)), or used to generate Schwann cells for treatment of spinal cord injury (McDonald et al., *Nat. Med.* 5: 1410 (1999)). The implementation of neural stem cell lines as a source material for brain tissue transplants is currently limited by the ability to induce specific neurochemical phenotypes in these cells (Wagner et al., *Nat. Biotechnol.* 17(7): 653, 1999). Specifically, there is a large unmet need for clinical cell implantation to patients suffering from PD/other neurodegenerative disorders. It would be very useful if there were accessible stem cells capable of differentiating into pure specific cell types, for example, DA neurons for clinical cell implantation to patients suffering from PD. Thus, what is required is a method for generating optimal cells for replacement, such as highly specialized human DA neurons that are capable of repairing an entire degenerated nigro-striatal system; homogeneous cells that can be reliably obtained and generated in sufficient numbers for a standardized medically effective intervention.

Summary of the Invention

In general, the invention provides a method to generate functional lineage-restricted progenitors from embryonic stem cells for obtaining donor cells of specific neuronal cell-fate, in sufficient quantities for the unmet cell transplantation need for treating patients with neurodegenerative diseases or disorders; for example, DA neural cells for the transplantation therapy of PD. In particular, the invention involves the selection of unmodified, totipotent embryonic stem cells derived from blastocysts, and inserting into these cells one or more cell-fate inducing genes, e.g., Nurr-1, PTX3, Phox 2a, AP2, Shh, that render them cell-fated to neurons. The ES cells are capable of differentiating under appropriate conditions to DA neurons, serotonergic neurons, astrocytes, Schwann cells, and/or oligodendrocytes. From differentiated ES cells, homogeneous cell populations of specific neuronal cell-fate are isolated by inserting a selectable marker gene cassette into a cell-specific gene expressed in a specific neuronal cell-type. For example, inserting a selectable gene cassette, e.g., β -geo (encoding for both neomycin resistance and β -galactosidase) into the dopamine transporter (DAT) or the tyrosine hydroxylase (TH) gene allows the selective isolation of DA neurons. These pure DA neurons are a useful source of donor cells for grafts into PD patients. Likewise, one can isolate serotonergic neurons from differentiated ES cells by inserting the same β -geo gene cassette into the tryptophan hydroxylase or the serotonin transporter gene that is expressed by serotonergic neurons or isolate astrocytes by inserting the β -geo gene cassette into the fibrillary acidic protein gene expressed by astrocytes. Specific lineage-restricted neural precursors thus can be isolated and expanded as a pure population, and used as donor cells in transplantation therapy of different neurodegenerative diseases, disorders, or abnormal physical states.

Accordingly, in a first aspect, the invention features a method of treating a human patient suffering from a neurodegenerative disease, including engrafting into a patient a population of ES recombinant cells that includes one or more cell fate-inducing genes that permit the cells to form neurons in the patient. Preferably, the cell fate inducing gene may be one or more of Nurr-1, PTX3, Phox 2a, AP2, and Shh. In one preferred embodiment, the one or more cell-fate inducing genes permit the cells to form DA neurons.

In a related aspect, the invention features a method of treating a human patient suffering from a neurodegenerative disease, wherein the cells are made by the steps of : a) obtaining one or more stem cells, b) transfecting one or more stem cells with one or more cell fate inducing genes, c) selecting one or more transfectants from step b), and d) expanding one or more selected transfectants from step c) to form a population of recombinant cells. Preferably, the step d) includes inducing cell division using a growth factor.

In another related aspect, the invention features a method of treating a human patient suffering from a neurodegenerative disease, wherein the cells are made by the steps of: a) obtaining one or more stem cells, b) expanding one or more stem cells, and c) transfecting multiple cells in the expanded cells from step b) with one or more cell fate inducing genes to form the population of recombinant cells. Preferably, step b) includes inducing cell division using a growth factor.

In preferred embodiments of each of the foregoing aspects of the invention, the cells are human unmodified, totipotent embryonic stem cells (TESCs). In other embodiments of the invention, the TESCOs can be from, for example, non-human primates, mice, and rats.

In preferred embodiments of each of the foregoing aspects of the invention,

the recombinant cells are a homogeneous cell population of a specific neuronal cell-type.

In preferred embodiments of each of the foregoing aspects of the invention, the one or more cell fate inducing genes cause the cells to form DA neurons. In other embodiments of the invention, the TESC's may, under appropriate conditions, differentiate into neurons, astrocytes, Schwann cells, and/or oligodendrocytes.

In preferred embodiments of each of the foregoing aspects of the invention, the growth factor used to expand the TESC's with or without the inserted genes for cell-fate induction is leukemia inhibitory factor ("LIF"). In other embodiments, a growth factor used to expand TESC's is basic fibroblast growth factor or epidermal growth factor.

TESC's can be stably or transiently transformed with a heterologous gene (e.g., one encoding a therapeutic protein, such as a protein which enhances cell divisions or prevents apoptosis of the transformed cell or other cells in the patient, or a cell fate-determining protein).

By "totipotent embryonic stem cell" or "TESC" is meant a cell that has the potential of differentiating into any type of cell. An embryonic stem cell is "totipotent" because it has the potential to differentiate into more than one cell type (e.g., a neuron, a skin cell, a hematopoietic cell).

The invention also features a pharmaceutical composition including (i) growth factor-expanded TESC's containing one or more cell-fate inducing genes, and (ii) a pharmaceutically acceptable carrier, auxiliary, or excipient.

Other features and advantages of the present invention will become apparent from the following detailed description and the claims. It will be

understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of example only, and various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the steps for ES cell procedures including *in vitro* expansion, chemical or spontaneous induction into neurons after implantation into the adult brain. Totipotent embryonic stem cells derived from the inner cell mass of blastocyst are propagated in culture in the presence of leukemia inhibitory factor (LIF). Prior to transplantation, LIF is removed, and the cells are then treated with retinoic acid (A) or are transplanted directly (B) into adult brain.

Figure 2 is a schematic representation of the steps involved in the non-linear trigger gene-induction of embryonic stem cells differentiating to donor neural cells, that are used for cell transfer/transplantation.

Figure 3A is the vector map of pIRES2-EGFP and Figure 3B is the vector map of pIRES2/EGFP/Nurr1 which expresses both the green fluorescent signal (EGFP) and dopamine-specific transcription factor Nurr1.

Detailed Description

The present invention provides a method to generate functional lineage-restricted progenitors from embryonic stem cells for obtaining pure cell populations of specific neuronal cell-fate; for example, DA progenitors for

obtaining donor DA neural cells in sufficient quantities for the unmet cell transplantation need for treating patients with neurodegenerative diseases or disorders. In particular, the invention features the selection of unmodified, TESC_s, and inserting these cells with one or more cell-fate inducing genes, e.g., Nurr-1, PTX3, Phox 2a, AP2, Shh, that render them cell-fated to neurons. TESC_s under appropriate conditions differentiate into DA neurons, Schwann cells, oligodendrocytes and/or astrocytes and can serve as donor cells for transplants to treat neurodegenerative diseases, disorders, or abnormal physical states. For example, the cells may be used as a source of DA neurons for grafts into PD patients. In one example, the cell-fate induction of TESC_s results in differentiated DA neurons which may be implanted in the substantia nigra or striatum of a PD patient. In a second example, the cells may be used to generate oligodendrocytes and/or astrocytes under appropriate conditions for use in transplants for the treatment of multiple sclerosis and other myelin-associated diseases. In still another example, the TESC_s may be used to generate Schwann cells for treatment of spinal cord injury. Using the genetic selection strategy as described in Example 7 *infra*, specific neuronal cell-types can be isolated as a homogeneous population and used as donor cells in transplantation therapy of these different diseases. Furthermore, in any of the foregoing examples, the cells may be modified to express, for example, a growth factor or other therapeutic compound, if desired.

Cell Therapy

The TESC_s of this invention may be used to prepare pharmaceutical compositions that can be administered to humans or animals for cell therapy. The cells may be undifferentiated or differentiated prior to administration. Dosages to be administered depend on patient needs, on the desired effect, and on the chosen

route of administration.

The invention also features the use of the cells of this invention to introduce therapeutic compound(s) into the diseased, damaged, or physically abnormal CNS, PNS, or other tissue. The TESC's thus act as a vector to deliver the compound(s). In order to allow for expression of other therapeutic compounds, suitable regulatory elements can be derived from a variety of sources, and may be readily selected by one of ordinary skill in the art. Examples of regulatory elements include a transcriptional promoter and enhancer or RNA polymerase binding sequence, and a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecule. The recombinant molecule may be introduced into the TESC's or the cells differentiated from the stem cells using *in vitro* delivery vehicles such as retroviral vectors, adenoviral vectors, DNA virus vectors, and liposomes. They may also be introduced into such cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as incorporation of DNA into liposomes. The genetically altered cells may be encapsulated in microspheres and implanted into or in proximity to the diseased or damaged tissue. Protocols employed are well-known to those skilled in the art, and may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997.

The methods of the invention can be used to treat any patient having a disease or disorder characterized by cell loss that can be ameliorated by administration of TESC's of the invention (or cells derived from these cells) to that patient. For example, TESC's may be used to generate DA neurons for use in transplants for the treatment of PD; oligodendrocytes and/or astrocytes

for use in transplants for the treatment of multiple sclerosis and other myelin-associated diseases; Schwann cells for treatment of spinal cord injury; DA neurons and/or serotonergic neurons for treatment of other neurodegenerative diseases or disorders such as Alzheimer's, Huntington's and Hirschsprung's disease. For uses of stem cells, also see Ourednik et al. (*Clin. Genet.* 56: 267, 1999), hereby incorporated by reference.

Example 1

TESC preparation

The mouse blastocyst-derived embryonic stem (ES) cell lines D3 and E14TG2a (A.T.C.C.; Rockland, MD) and B5 (Hadjantonakis et al., *Mech. Dev.* 76: 79 (1998) were used for all studies (Doetschman et al., *J. Embryol. Exp.* 87: 27-45, 1985; Finger et al., *J. of Neurol. Sci.* 86: 203-213); the E14TG2a line was HPRT-deficient. All ES cell lines were propagated and maintained as described (Deacon et al., *Experimental Neurology* 149: 28 (1998)). Undifferentiated ES cells were maintained on gelatin coated dishes in Dulbecco's modified Minimal Essential Medium (DMEM, Gibco/BRL, Grand Island, NY) supplemented with 2mM glutamine (100X stock from Gibco/BRL), 0.001% β -mercaptoethanol, 1X non-essential amino acids (100X stock from Gibco/BRL), 10% donor horse serum (HyClone, Logan, UT), and human recombinant leukemia inhibitory factor (LIF; R & D Systems, Minneapolis, MN) (Abercrombie, *M. Anat. Rec.* 94, 239-247 (1946)). Early passage cultures were frozen (90% horse serum/10% DMSO), thawed for use, and cultured for two weeks in the presence of LIF. Cells were trypsinized (0.05% trypsin-EGTA; GIBCO), resuspended, then seeded at 1.5×10^6 cells in 5ml of DMEM + 0.5 mM retinoic acid (RA+) (Sigma Chemical Co., St.

Louis, MO) or in the same media without RA (RA-) in a 60 mm Fisher brand bacteriological grade petri dish, in the absence of LIF. Horse serum was replaced by 10% fetal calf serum (FCS; Hyclone) during this treatment. ES cells did not adhere to the dish but formed small aggregates (embryoid body). After 2 days of incubation at 37°C, the cells were transferred to a 15 ml sterile culture tube and allowed to settle, and the media was replaced with an equal volume of fresh RA+ or RA- media. The cells were then re-plated and incubated for an additional 2 days. After 4 days, cells were collected and rinsed once in Ca²⁺ and Mg²⁺-free Dulbecco's Phosphate-Buffered Saline (D-PBSa, Gibco/BRL). D-PBSa was removed, 0.5 ml of trypsin solution was added, and the cells were incubated for 5 minutes at 37°C, then triturated with a pasteur pipette to dissociate the cells. The trypsin solution was replaced with 0.1 M phosphate buffered saline pH 7.4 (PBS), and viability was determined by the acridine orange-ethidium bromide method (Brundin, P., et al., *Brain Res.* 331, 251-259 (1985)); viability of cells after removal from the culture dish was greater than 95% in all cases. ES cells derived directly from monolayers after LIF removal were also implanted in some cases, following the above procedures minus the incubation steps. No systematic difference due to incubation time was observed in the resulting grafts and so RA- cases are pooled in this report (see Figure 1 for schematic showing basic steps for ES cell procedures).

Example 2

Genetic modification of mouse blastocyst-derived ES cells

By way of example, construction of a Nurr1 expressing ES cell line is described. Nurr1 cDNA was subcloned into the SacI site in pIRES2-EGFP (Clontech)[see Figures 3A and 3B]. Nurr1- containing plasmids were amplified

in *E. coli* and purified with the QIAGEN plasmid purification kit (QIAGEN Inc.). The construct's functionality was tested by demonstrating its ability to induce tyrosine hydroxylase (TH) reporter gene expression in cell lines such as BE(2)C cells, followed by β -galactosidase and CAT-assays. pIRES2-EGFP with [see Figure 3B] and without Nurr1 insert [see Figure 3A] was linearized with Afl II and isolated after 1% agarose gel electrophoresis for transfection to embryonic stem (ES) cells.

ES D3 cells were seeded into gelatin coated dishes to an approximate confluence of 25%. Next morning, the cells were transfected using Lipofectamin PLUS (GIBCO BRL, Life technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. [30 μ g DNA in 750 μ l serum free media and 60 μ l PLUS were mixed and incubated at RT for 15 minutes after which 60 μ l Lipofectamin in 750 μ l serum free media was added and the mixture incubated for another 15 minutes at RT. The mixture was added drop-wise to cultured cells in a 100mm dish containing 5 ml ES-media (450ml high glucose DMEM, 50ml horse serum (HS), 5ml 100x L-glutamine, 5ml Hees, 5ml 100x NEAR, 5ml β -mercaptoethanol and 100 l. LIF 30 μ g/ml).]

After 24th, 5ml fresh ES-media was added and after another 6th cells were split and cultured in ES media containing 500 μ g/ml Neomycin (G418 Sulfate, Clontech Palo Alto, CA, USA) for selection. Leftover cells were frozen in ES-freezing media (90% horse serum and 10% DMSO). The concentration of Neomycin needed for selection was determined by culturing untransfected and transfected cells in a range of titers of Neomycin.

Cells split 30h after transfection were pooled together, cell stocks were made, and cells were cultured to be used for RT-PCT analysis and immunocytochemistry. Fresh transfected cells (frozen 30h after transfection) were

thawed and seeded, highly diluted, in gelatin coated dishes and grown for five days in ES-media with G418 (500µg/ml). Well-isolated colonies were picked using cloning cylinders and cloning discs and transferred to a gelatin coated 24 well plate. Cells were grown to confluency (between 10 and 14 days), harvested and frozen in 0.5 ml ES-freezing media. A small number of the cells (~1/8) were expanded for RNA preparation. Clones were screened to detect Nurr1-expression, using GeneAmp ThermoStable rTth Reverse Transcriptase RNA PCT Kit (PERKIN ELMER, Branchburg, NJ, USA) according to the manufacturer's protocol.

Multiple Nurr1-expressing ES cell lines isolated after Neomycin selection were used for *in vivo* transplantation as well as *in vitro* differentiation into the DA phenotype. Differentiation of neural stem cells into DA neurons requires overexpression of Nurr1 as well as a factor derived from local type 1 astrocytes (see Wagner et al., *Nat. Biotechnol.* 17(7): 653, (1999)). Hence, these Nurr1 expressing ES cells can also serve as a source of DA neurons. Protocols employed here are well-known by those skilled in the art and may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997.

These non-human primate ES cell lines provided an accurate *in vitro* model for human transplantation studies.

Example 3

In vitro differentiation of naive and transgenic ES cell lines

The method of differentiating ES cells into neural progenitor cells and into

DA and serotonergic neurons *in vitro* has been reported (Lee et al., *Nat. Biotechnol.* 18: 675, (2000)). This procedure was adapted for D3 and B5 ES cells and further modified for Nurr1-expressing transgenic ES cell lines. Briefly, D3 and B5 ES cells were differentiated into embryoid bodies (EBs) in suspension culture for four days after removal of leukemia inhibitory factor (LIF). The EBs are then plated onto adhesive tissue culture surface in the ES cell differentiation medium. After 24 hr of culture, nestin-positive cells were selected by replacing the medium by serum-free ITSFn medium (Rizzino and Crowley, *Proc. Natl. Acad. Sci.* 77: 457, (1980)); Okabe et al., *Mech. Dev.* 59: 89, (1996)). After 6-10 days of selection, nestin-positive cells were expanded by dissociating the cells by trypsinization and subsequent plating on tissue culture plastic containing N2 medium (Johe et al., *Genes Dev.* 10:129, (1996)) supplemented with laminin (1mg/ml) and bFGF (10 ng/ml). After expansion for six days, the medium was changed every two days. Differentiation was induced by removal of bFGF from the medium. Signaling molecules known to induce the TH⁺ phenotype, *e.g.*, analog of cAMP, retinoic acid, Shh, FGF8, and ascorbic acid (Kalir and Mytilineou, *J. Neurochem.* 57: 458, (1991); Kim et al., *Proc. Natl. Acad. Sci.*, (1993); Lee et al., *Nat. Biotechnol.* 18: 675, 2000) were used and compared in naive and transgenic ES cell lines. Expression of marker expression was examined by immunocytochemistry and RT-PCR analysis. To determine the molecular changes between nestin-positive neural progenitor cells and more differentiated TH⁺ neurons, EBs were collected from each stage of *in vitro* differentiation as described above. Poly (A)⁺ RNA were isolated and the probes prepared subsequently.

Example 4

ES cell transplantation

Sprague-Dawley rats (300-350g) and C57/B15 mice (14-17g) (Charles River Labs, MA) were used as intracerebral-transplant recipients. Cell concentrations and dosages varied in different experiments: rat hosts received from 100,000 to 300,000 viable ES cells per right striatum (60,000-100,000 viable cells/l.), and mice received 60,000 ES cells per right striatum (60,000 viable cells/l.). For all neural surgical procedures, animals were anesthetized with pentobarbital (65 mg/kg, i.p.), and placed in a Kopf stereotaxic frame (with Kopf mouse adapter for mice). Mice (n=7) used as intracerebral transplant hosts were normal adult females, and rats (n=31) used as transplant hosts were adult females that had received prior unilateral nigrostriatal 6-hydroxydopamine (6-OHDA) lesion removing at least 97% of DA innervation, as previously described (Galpern et al., *Cell Transplant.* 140 :1-13, (1996)). ES cells were implanted stereotactically (from Bregma: A+ 1.0 mm, L -2.5 mm, V -4.5 mm; IB -2.5 mm). A 10 l. Hamilton syringe attached to a 22S-gauge needle (ID/OD 0.41 mm/0.71 mm) was used to deliver 1 l. (mouse) or 3-5 l. (rat) of ES cell suspension (rate: 1 ml/min, allowing an additional 2 min for the final injection pressure to equilibrate before slowly withdrawing the injection needle). Starting on the day prior to transplantation, rats were immunosuppressed with Cyclosporine-A (CsA, Sandimmunne, MA)(10-15 mg/kg, s.c. daily) diluted in extra virgin olive oil for the duration of the experiment to prevent graft rejection. CsA blood levels were assayed each week (Quest Diagnostics, MA).

Mice were not immunosuppressed. Nude mice (Charles River) were used as kidney-capsule transplant recipients. Mice were anesthetized (as above), and 50,000 ES cells (in 1 ml), not pre-treated with RA, were injected into a blood clot derived from host blood; this clot was then implanted unilaterally into one kidney capsule (n=3 with E14TG2a line and n=3 with D3 line). (See Figure 2 for

schematic showing the various steps involved in the non-linear gene induction of embryonic stem cells differentiating to donor neural cells that are used for transplantation)

Histological procedures

Two or four weeks after transplantation, animals were terminally anesthetized (pentobarbital; 100mg/kg), then perfused intracardially with 100 ml heparin saline (0.1% heparin in 0.9% saline), followed by 400 ml of paraformaldehyde (4% in PBS). The brains or kidney capsules were removed and post-fixed for 8 hours in the same 4% paraformaldehyde solution. Following post-fixation, the brains and kidney capsules were equilibrated in sucrose (30% in PBS), sectioned (40 μ m) on a freezing microtome, and collected in PBS. Sections were divided into 6-8 series and stored in PBS at 4°C. Separate series were processed for either Nissl staining (cresyl violet acetate), or acetylcholinesterase (AChE) histochemistry (as described in Pakzaban et al., *Exp. Brain Res.* 97: 13-22). Immunohistochemical markers used for tissue processing included antibodies directed against neuron-specific enolase (NSE, Dako, Carpinteria, CA), mouse-specific Thy 1.1 (Clone TN-26, Sigma), tyrosine hydroxylase (TH; PelFreez, Rogers, AK), 5-hydroxytryptamine (5-HT, Arnel Products, New York, NY), 200kD + 68kD neurofilament (NF, Biodesign, Kennebunkport, ME), dopamine- β -hydroxylase (DBH; Chemicon, Temecula, CA), proliferating cell nuclear antigen (PCNA; Chemicon), and glial fibrillary acidic protein (GFAP; Boehringer-Mannheim).

Free floating tissue sections were pretreated with 50% methanol and 3% hydrogen peroxide in PBS for 20 minutes, washed 3 times in PBS, and incubated in 10% normal goat serum (NGS) in PBS for 60 minutes prior to overnight

incubation on a shaking platform with the primary antibody. After a 10-minute rinse in PBS and two 10-minute washes in 5% NGS, sections were incubated in biotinylated secondary antibody (goat-anti-rabbit or goat-anti-mouse, depending on primary species) at a dilution of 1:200 in 2% NGS in PBS at room temperature for 60-90 min. The sections were then rinsed three times in PBS and incubated in avidin-biotin complex (Vectastain ABC Kit ELITE; Vector Labs) in PBS for 60-90 min at room temperature. Following thorough rinsing with PBS and Tris-buffered saline, sections were developed for 5-30 min in 0.04% hydrogen peroxide and 0.05% 3, 3'-diaminobenzidine (Sigma) in Tris-buffered saline. Controls with omission of the primary antibody were performed on selected sections to verify the specificity of staining. After immunostaining, floating tissue sections were mounted on glass slides, coverslipped, and analyzed with bright and darkfield light microscopy using a Zeiss Axioplan microscope. Quantitative analyses were performed with the aid of NIH Image software (Ray Rasband, NIH, Bethesda, MD) and cell counts from serial sections were corrected and extrapolated for whole graft volumes using the Abercrombie method (Finger, S., et al., *Journal of Neurological Sciences* 86, 203-213 (1988)). Selected images were digitized using a Leaf Lumina video scanning camera (Leaf Systems, Newton, MA) into Adobe Photoshop which was used to prepare and print final figures.

Example 5

Embryonic stem cell lines derived from human blastocysts

Fresh or frozen cleavage stage human embryos, produced by *in vitro* fertilization (IVF) were cultured to the blastocyte stage in G1.2 and G2.2 medium. These embryos were donated by individuals after informed consent and after

institutional review board approval. 14 inner cell masses were isolated by immunosurgery, with a rabbit antiserum to BeWO cells, and plated on irradiated (35 grays gamma irradiation) mouse embryonic fibroblasts. Culture medium consisted of 80% Dulbecco's modified Eagle's medium (no pyruvate, high glucose formulation; Gibco-BRL) supplemented with 20% fetal bovine serum (Hyclone), 1mM glutamine, 0.1 mM β -mercaptoethanol (Sigma), and 1% nonessential amino acid stock (Gibco-BRL). After 9-15 days, the inner cell mass-derived outgrowths were dissociated into clumps either by exposure to $\text{Ca}^{2+}/\text{Mg}^{2+}$ free phosphate-buffered saline with 1mM EDTA, by exposure to dispase, or by mechanical dissociation with a micropipette and replated on irradiated mouse embryonic fibroblasts in fresh medium. Individual colonies with a uniform undifferentiated morphology were individually selected by micropipette, mechanically dissociated into clumps, and replated. Once established and expanded, cultures were passaged by exposure to type IV collagenase (1 mg/ml; Gibco-BRL) or by selection of individual colonies by micropipette. Clump sizes of about 50-100 cells were optimal. The resulting cells had a high ratio of nucleus to cytoplasm, prominent nucleoli, and a colony morphology similar to that of rhesus monkey ES cells. Cell lines can be cryopreserved and thawed when required. Continuous culturing does not lead to a period of replicative crisis in the cell lines (For details, see Thompson et al., *Science* 282 (5391): 1145 (1998), incorporated herein by reference). Also see Vescovi et al., *J. Neurotrauma* 16(8): 689 (1999); Vescovi et al., *Exp. Neurol.*, 156(1): 71 (1999); Brustle O et al., *Science* 285(5428): 754 (1999) for methods for isolation and /or intracerebral grafting of non-transformed embryonic human stem cells.

Example 6

Transformation of human TESC's

In therapy for neurodegenerative diseases, it is desirable to transplant cells that are genetically modified to survive the insults that caused the original neurons to die. In addition, TESC's may be used to deliver therapeutic proteins into the brain of patients with neurodegenerative disorders to inhibit death of host cells.

According to the invention, TESC's are induced to differentiate into a desired cell type by transfecting the cells with nucleic acid molecules encoding proteins that regulate cell fate decisions (e.g., transcription factors such as Nurr-1, PTX3, Phox2a, AP2, and Shh). Nurr1 is known to regulate the development of midbrain dopaminergic neurons (Zetterstrom et al., *Science* 276: 248, (1997)). Our studies further indicated that Nurr1 may control dopaminergic fate by directly transactivating TH gene transcription. Ptx3 is another transcription factor specifically expressed in dopaminergic neurons but its precise function is not clear as yet (Smidt et al., *Proc. Natl. Acad. Sci.* 94:13305, (1997); Smidt et al., *Nat. Neurosci.* 3: 337, (2000)). Recent studies have showed that Phox2a is critical for both the development and neurotransmitter identity of noradrenergic neurons (Morin et al., *Neuron* 18: 411, (1997); Yang et al., *J of Neurochem.* 71:1813, (1998)). Shh is a signaling molecule which has been shown to be critical for determining the development of both the dopaminergic and serotonergic neurons (Ye et al., *Cell* 93: 755, (1998)). Our recent analysis also indicated that AP2 may control both the TH and dopamine β -hydroxylase promoter activities and thus regulate catecholamine production. Using such a method, it is possible to induce the differentiation of the specific cell types required for transplant therapy. Recombinant adenoviral vectors can be used to manipulate both postmitotic sympathetic neurons and cortical progenitor cells, with no cytotoxic effects.

Blastocyst-derived TESC's were transfected with a recombinant, attenuated adenovirus carrying the β -galactosidase reporter gene inserted in the deleted E1

region. Multiplicity of infection (MOI) was calculated based on titration on cells for adenovirus-based vectors, and represents the number of plaque-forming units added per cell. Staining for expression of the β -galactosidase marker gene was performed. Cells were fixed with 0.2% glutaraldehyde in PBS (pH 7.4) for 15 minutes at 4°C. After two washes with PBS, cells were incubated for 18 hours in X-gal stain (2 mM MgCl_2 , 1 mg/ml X-gal, 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, and 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ in PBS (pH 7.4). To estimate the percentage of cells that were infected, the total cell number and lacZ-positive cells can be counted in five random fields.

Similar Adenovirus vectors, carrying different regulatory cell-fate inducing genes including Nurr1, PTX3, Phox2a, AP2, and/or Shh, are constructed and used to express their gene products in TESC. Expression of these genes is monitored by Northern Analysis, Western Analysis and/or Immunohistochemical analysis. Protocols for the same may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997 and in *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Details of the cell-fate inducing genes can be accessed at: <http://www.ncbi.nlm.nih.gov/Pubmed/>: The National Center for Biotechnology Information; see below for Genebank Accession Numbers.

<u>Cell-fate inducing gene</u>	<u>Genebank accession number</u>
Shh(human)	NM 000193
AP-2(human)	X77343

Phox2a(human)	NM 003924
Phox2a1(human)	NM 005169
PTX3(Rat)	AJ011005
PTX3(human)	X6306
Nurr1(human)	AB017586
Nurr1(Rat)	U72345
Nurr2(Mouse)	ABO14889

Example 7

Selection of homogeneous cell populations of specific neuronal cell-fate from differentiated ES cells

ES cells can differentiate into various cell types *in vitro* by exposure to different extracellular signaling molecules. By combining several signaling molecules known to induce the DA neuronal cell-fate, a recent study reported that more than 20% of the cell population were induced to differentiate into tyrosine hydroxylase (TH)-positive cells (see Lee et al., *Nat. Biotechnol.* 18: 675 (2000)). However, these cell populations still contained various other different cell-types including serotonergic neurons and glial cells. At present, it is uncertain whether these mixed population of ES-derived cells are an optimal source of donor cells in transplantation therapy. Hence, we developed a strategy to selectively isolate homogenous cell populations with specific neuronal cell-fate; in particular, the DA cell-fate. A recent study showed that neuroepithelial cells can be efficiently selected from differentiated ES cells by inserting a selectable marker gene into the

Sox2 gene that is specifically expressed in neuroepithelial cells (Li et al., *Curr. Biol.* 8:971 (1998)).

For DA neurons, dopamine transporter (DAT) is another specific marker protein in addition to that of TH. Introduction of a selectable marker/reporter gene cassette into the DAT or TH gene of ES cells allows the selective isolation of a homogenous cell population of DA neurons. Similarly, one can isolate a pure population of serotonergic neurons by inserting the selectable gene cassette into the tryptophan hydroxylase or serotonin transporter gene. This selection strategy can be employed in other cell-types, by introducing the selectable gene cassette into a gene known to be expressed in specific neuronal cell-types (*e.g.*, the glial fibrillary acidic protein gene for isolating astrocyte cells).

Thus, to isolate the desired lineage-specific neural progenitors, plasmid constructs will be made in which the bifunctional selection marker/reporter gene cassette β -geo [coding for both the β -galactosidase and the neomycin resistance gene; see Friedrich G and Soriano P, *Genes Dev.* 5: 1513, (1991)] will be cloned into the cell-specific gene of interest in ES cells, such that the β -galactosidase and the neomycin phosphotransferase genes are expressed in a cell-specific manner. At the 3' end of the cell-specific gene, a phosphoglycerate kinase-hygromycin (pGK-hygro) resistant gene will be cloned (see Mortensen RM et al., *Mol. Cell. Biol.* 12:2391, (1992)). The plasmid will be cut with restriction enzymes to linearize a fragment containing the 5' region of the cell-specific gene β -geo cassette-pGK-hygro cassette-3' sequence of the cell-specific gene. The linearized fragment will be electroporated into ES cells (see Klug MG et al., *J. Clin. Invest.* 98 :21, (1996); Li ML et al., *Curr. Biol.* 8: 971, (1998). Transfected clones will be selected by growth in the presence of 200 μ g/ml hygromycin (Calbiochem, La Jolla, CA). Transfected ES cells will be cultured (see Smith AG et al., *J Tissue*

Culture Methods 13: 89, (1991)) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) (GIBCO/BRL), 1% nonessential amino acids (GIBCO/BRL), 0.1 mmol/l 2-mercaptoethanol (GIBCO/BRL), 1 mmol/l sodium pyruvate, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. The undifferentiated state will be maintained by 1,000 U/ml recombinant leukemia inhibitory factor (LIF) (GIBCO/BRL). To induce differentiation, hygromycin resistant ES cells will be plated onto a 100-mm bacterial Petri dish containing 10 ml of DME lacking supplemental LIF. After 3 d in suspension culture, the resulting embryoid bodies will be plated onto plastic 100-mm cell culture dishes and allowed to attach. The differentiated cultures will be grown in the presence of G418 (200 µg/ml; Gibco Laboratories, Grand Island, NY), resulting in selection of cell-specific ES cells. Expression of cell-specific genes is monitored by Northern Analysis, Western Analysis and/or Immunohistochemical analysis. Protocols for the same may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997 and in *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Details of the cell-specific genes can be accessed at : <http://www.ncbi.nlm.nih.gov/Pubmed/>: The National Center for Biotechnology Information; see below for Genebank Accession Numbers.

<u>Neuronal cell-type</u>	<u>Cell-specific gene(human)</u>	<u>Genebank accession number</u>
DA neurons	dopamine transporter(DAT)	D88570
DA neurons	tyrosine hydroxylase(TH)	D00292
serotonergic neurons	tryptophan hydroxylase	X83213

serotonergic neurons	serotonin transporter	AF117826
astrocytes	glial fibrillary acidic protein	BE222981

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Other embodiments are within the claims.

What is claimed is:

1. A method of treating a human patient suffering from a neurodegenerative disease, said method comprising:

engrafting into said patient a population of recombinant cells comprising one or more cell fate-inducing genes that permit said cells to form neurons in said patient.

2. The method of claim 1, wherein said cell-fate inducing genes are one or more of Nurr-1, PTX3, Phox 2a, AP2, and Shh.

3. The method of claim 1, wherein said cells are made by the steps of:

- a) obtaining one or more stem cells,
- b) transfecting said one or more stem cells with said one or more cell fate inducing genes,
- c) selecting one or more transfectants from step b), and
- d) expanding said one or more selected transfectants from step c) to form said population of recombinant cells.

4. The method of claim 3, wherein step d) comprises inducing cell division using a growth factor.

5. The method of claim 4, wherein said growth factor is leukemia inhibitory factor.

6. The method of claim 1, wherein said cells are made by the steps of:

a) obtaining one or more stem cells,

b) expanding said one or more stem cells, and

c) transfecting multiple cells in the expanded cells from step b) with said one or more cell fate inducing genes to form said population of recombinant cells.

7. The method of claim 6, wherein step b) comprises inducing cell division using a growth factor.

8. The method of claim 7, wherein said growth factor is leukemia inhibitory factor.

9. The method of claim 1, wherein said one or more cell fate inducing genes permit said cells to form dopaminergic neurons.

10. The method of claim 1, wherein said recombinant cells are a homogenous cell population of a specific neuronal cell-type.

11. The method of claim 10, wherein said one or more cell fate inducing genes permit said cells to form dopaminergic neurons.

ABSTRACT OF THE DISCLOSURE

Disclosed herein is a method for generating functional lineage-restricted progenitors from embryonic stem cells for obtaining donor cells of specific neuronal cell-fate, in sufficient quantities for the unmet cell transplantation need for treating patients with neurodegenerative diseases or disorders.

Figure 1

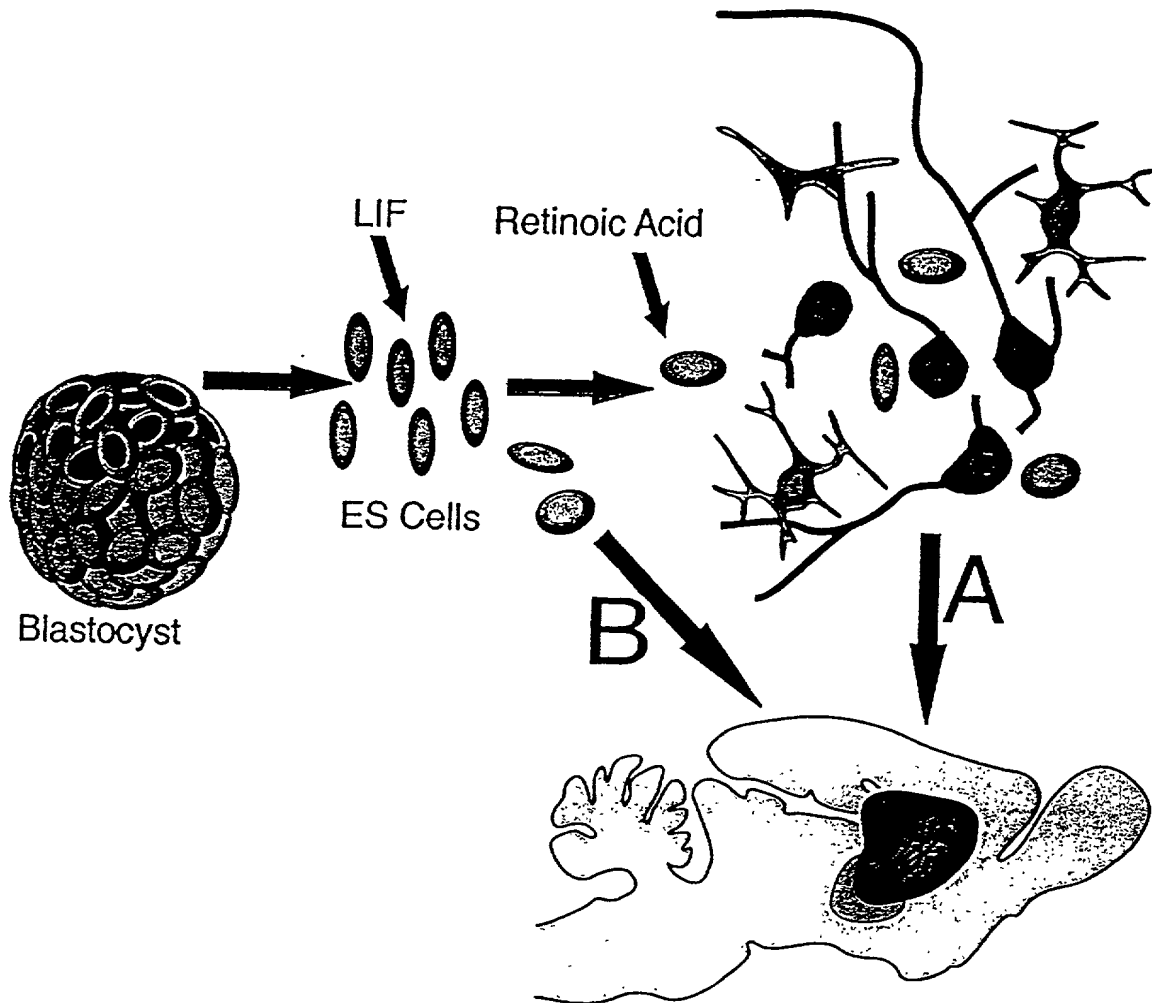


Figure 2

NON-LINEAR TRIGGER GENE INDUCED CELL FATE

OLE ISACSON

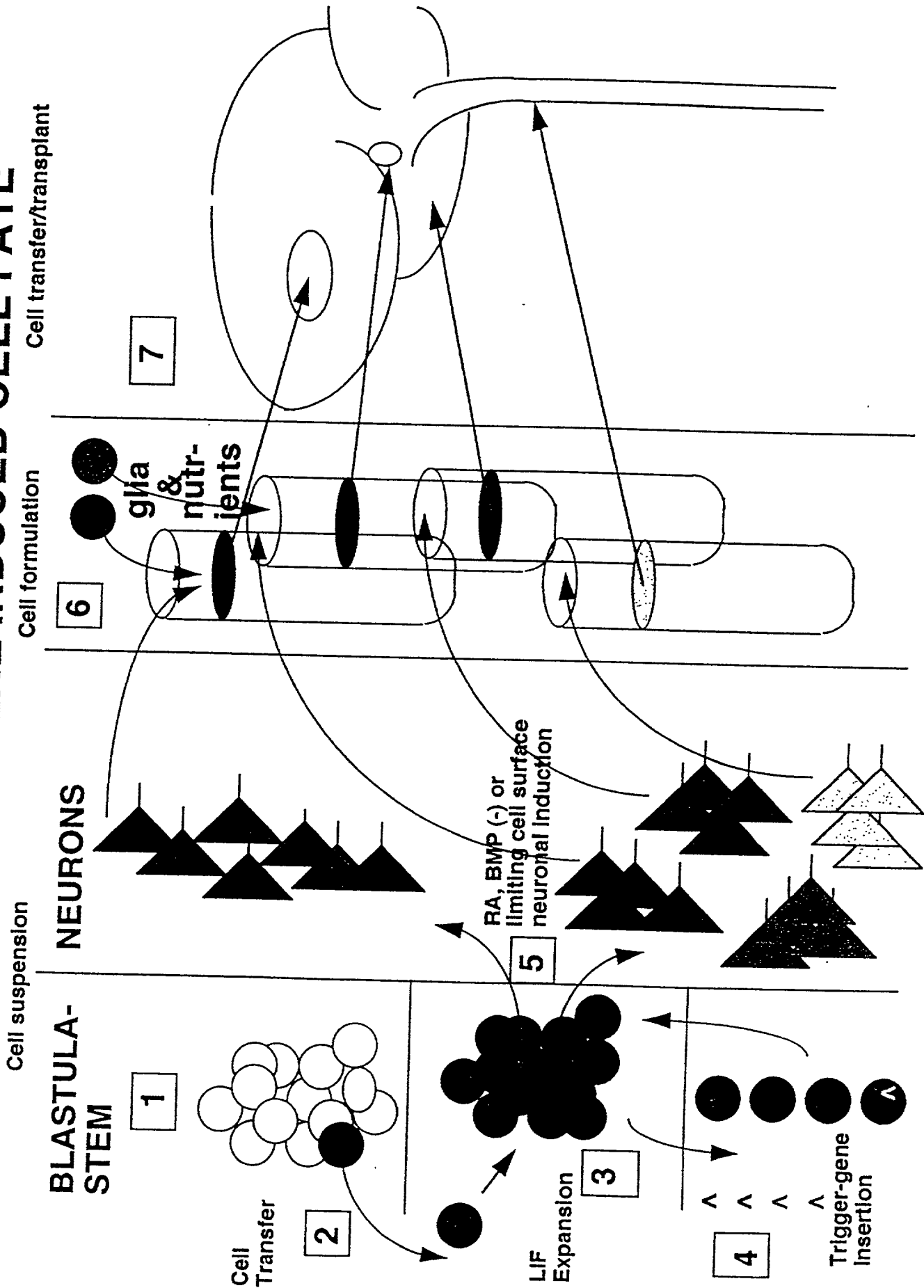
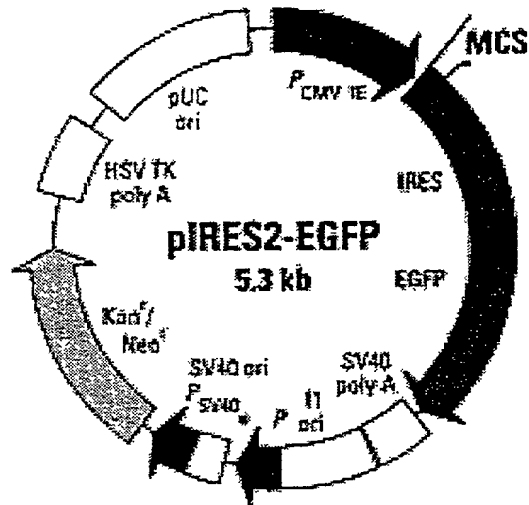


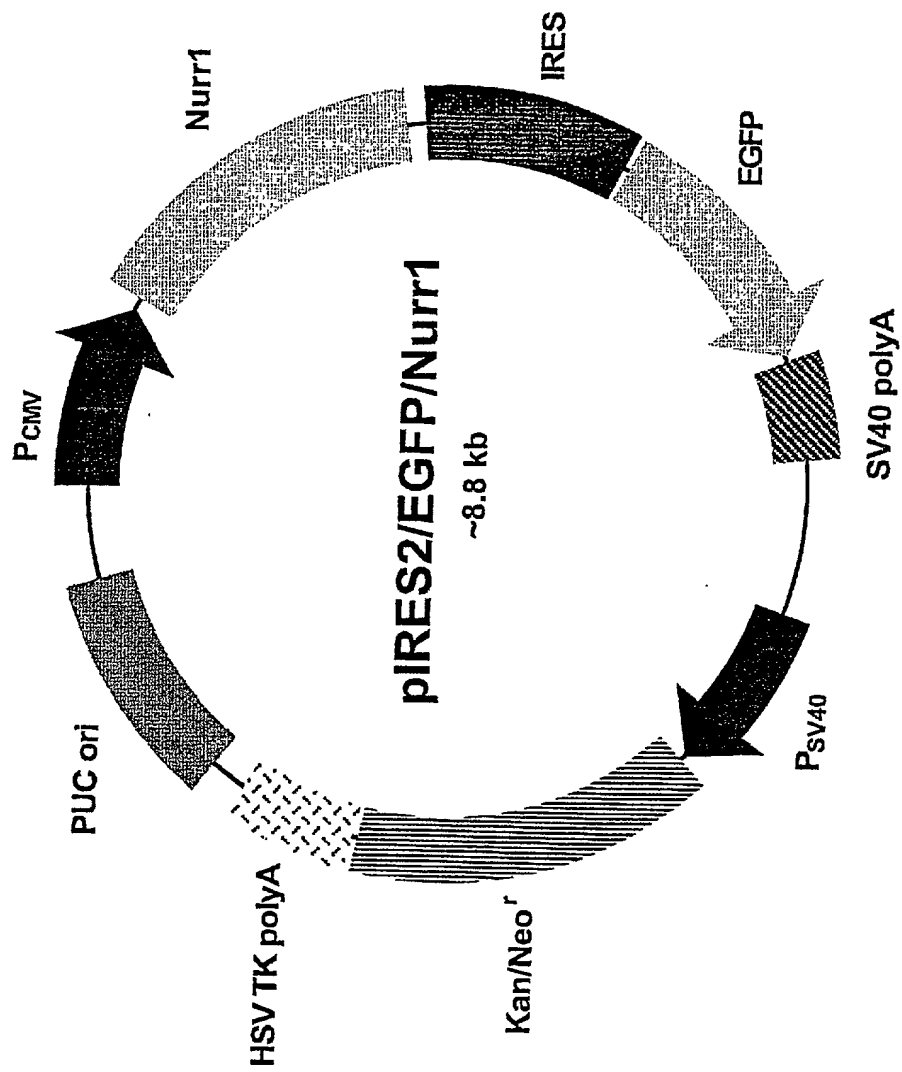
Figure 3A

Vector map of pIRES2-EGFP.

Picture from <http://www.clontech.com/techinfo/vectors/pIRES2-EGFP.html>. Accessed 5/18/00.
Additional information about the vector can be accessed from that site.



00223355 Figure 3B



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled CELL IMPLANTATION THERAPY FOR NEURODEGENERATIVE DISEASES OR DISORDERS, the specification of which

- ☒ is attached hereto.
☐ was filed on _____ as Application Serial No. _____
and was amended on _____.
☐ was described and claimed in PCT International Application No. _____
filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

FOREIGN PRIORITY RIGHTS: I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
			Yes/No

PROVISIONAL PRIORITY RIGHTS: I hereby claim priority benefits under Title 35, United States Code, §119(e) and §120 of any United States provisional patent application(s) listed below filed by an inventor or inventors on the same subject matter as the present application and having a filing date before that of the application(s) of which priority is claimed:

Serial Number	Filing Date	Status

NON-PROVISIONAL PRIORITY RIGHTS: I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by

COMBINED DECLARATION AND POWER OF ATTORNEY

the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Filing Date	Status

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bieker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885, Mary Rose Scozzafava, Ph.D., Reg. No.36,268, James D. DeCamp, Ph.D., Reg. No. 43,580, Sean J. Edman, Reg. No. 42,506, Timothy J. Douros, Reg. No. 41,716.

Address all telephone calls to: Paul T. Clark at 617/428-0200.

Address all correspondence to: Paul T. Clark at Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name (First, Middle, Last)	Residence Address (City, State, Country)	Post Office Address (Street, City, State, Country)	Citizenship
Ole Isacson			
Signature:			Date:

Full Name (First, Middle, Last)	Residence Address (City, State, Country)	Post Office Address (Street, City, State, Country)	Citizenship
Kwang Soo Kim			
Signature:			Date: